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TECHNICAL MANUSCRIPT 54

OBSERVATIONS ON A PHENOTYPIC MODIFICATION OF VARIOLA VIRUS IN TISSUE CULTURE

DECEMBER 1963

UNITED STATES ARMY
BIOLOGICAL LABORATORIES
FORT DETRICK

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AD 428033

THE FOLLOWING PAGES ARE CHANGES

TO BASIC DOCUMENT

ERRATUM SHEET

SUBJECT REPORT: Technical Manuscript 54
"Observations on a Phenotypic Modification of Variola Virus in Tissue Culture"

DATE ISSUED: December 1963

CORRECTION: Please substitute the following in place of page 3.

ABSTRACT

Pirsch et al (Bact. Proc., 1961) reported that a qualitative difference between cell-associated virus (INV) and supernatant virus (EXV) was evident immediately after variola virus was passaged in guinea pig lung (GPL) cells. INV could be continuously passaged efficiently (p+ = passageability) but EXV of even higher initial titer could not (p-). As EXV was passaged and titers declined as expected, INV, taken at the different EXV passage levels, proved to be p+; thus, the initial change from p+ to p- upon release from GPL cells formally resembles a host-induced modification, except that a single host produces both phenotypes. However, to regain p+ (when titers rise), one additional passage was required during which the normal decline in titer was arrested and the virus concentration merely maintained. This suggests that some priming in the cell was necessary before virus titer could increase significantly, a notion now under study. An analysis was made to define the steps in the growth cycle where the qualitative difference between INV and EXV is reflected. Thus far, results show that adsorption and/or penetration is greater in extent and occurs more rapidly with INV than with EXV. Further, INV has a shorter lag phase, and is released into the medium more rapidly and to a significantly higher titer than EXV. It is concluded that EXV is p- because relatively little is released for infection of new cells, and what is released is of relatively poor infectious quality. The same observation was made with vaccinia virus, but the change from p+ to p- was more gradual.

AD 428033

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U.S. ARMY BIOLOGICAL LABORATORIES
Fort Detrick, Frederick, Maryland

TECHNICAL MANUSCRIPT 54

OBSERVATIONS ON A PHENOTYPIC MODIFICATION OF VARIOLA VIRUS IN TISSUE CULTURE

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Project 1A012501B028 December 1963

A portion of the work reported here was performed under Project 4X99-26-001, "Basic Research in Life Sciences," Task 02, "Microbiology." The expenditure order was 2039. This material was originally submitted as manuscript 5100.

ABSTRACT

Pirsch et al (Bact. Proc., 1961) reported that a qualitative difference between cell-associated virus (EXV) and supernatant virus (INV) was evident immediately after variola virus was passaged in guinea pig lung (GPL) cells. EXV could be continuously passaged efficiently (p+ = passageability) but INV of even higher initial titer could not (p-). As INV was passaged and titers declined as expected, EXV, taken at the different INV passage levels, proved to be p+; thus, the initial change from p+ to p- upon release from GPL cells formally resembles a host-induced modification, except that a single host produces both phenotypes. However, to regain p+ (when titers rise), one additional passage was required during which the normal decline in titer was arrested and the virus concentration merely maintained. This suggests that some priming in the cell was necessary before virus titer could increase significantly, a notion now under study. An analysis was made to define the steps in the growth cycle where the qualitative difference between EXV and INV is reflected. Thus far, results show that adsorption and/or penetration is greater in extent and occurs more rapidly with EXV than with INV. Further, EXV has a shorter lag phase, and is released into the medium more rapidly and to a significantly higher titer than INV. It is concluded that INV is p- because relatively little is released for infection of new cells, and what is released is of relatively poor infectious quality. The same observation was made with vaccinia virus, but the change from p+ to p- was more gradual.

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I. INTRODUCTION

Pirsch and colleagues have reported[1] that cell-associated (or intracellular) variola virus from guinea pig lung (GPL) cells could be passaged efficiently in those cells as measured in pock-forming units, but that supernatant (or extracellular) virus could not be passaged and continually declined in titer under the same conditions. Attempts to modify either virus preparation by treatment with trypsin or with fluorocarbon have not been successful. Our present experimental findings enable us to more clearly define the qualitative differences between intracellular and extracellular virus and indicate that release of the virus through the cell membrane of GPL cells induces a reversible phenotypic change in the virus populations found in the supernatant.

II. MATERIALS AND METHODS

Variola virus was assayed by means of pock counts on the chorioallantoic membrane (CAM) of 11- to 12-day-old embryonated eggs. An alternative method used for special experiments was one described recently by Pirsch and Purlson,[2] based on the enumeration of hyperplastic foci formed on monolayers of HeLa cells.

The HeLa cells, and a line of GPL cells originally isolated in our laboratories several years ago, were grown in medium 199 supplemented with calf serum and containing penicillin and streptomycin. Cell monolayers were washed with Hanks' balanced salt solution (BSS) and for the initial passage a 1:10 dilution of a 10 per cent CAM suspension of variola virus was used in the GPL cells. From then on, intracellular and extracellular viruses were passed every two days.

Extracellular virus was obtained by removing the overlying medium, which was centrifuged to remove the cell debris. The intracellular virus was obtained by trypsinizing infected GPL cells from T-60 Earle flasks, followed by low-speed centrifugation, removal of the supernatant, and liberation of virus from the cells by sonic vibration or repeated cycles of freezing and thawing.

III. RESULTS

Figure 1 recalls the data[2] showing that intracellular virus can be passaged efficiently although extracellular virus cannot.

A number of experiments were performed in an effort to see whether the failure of supernatant virus to pass could be explained on the basis of interference. Table I shows that viral interference or interferon was not demonstrable in our experiments.

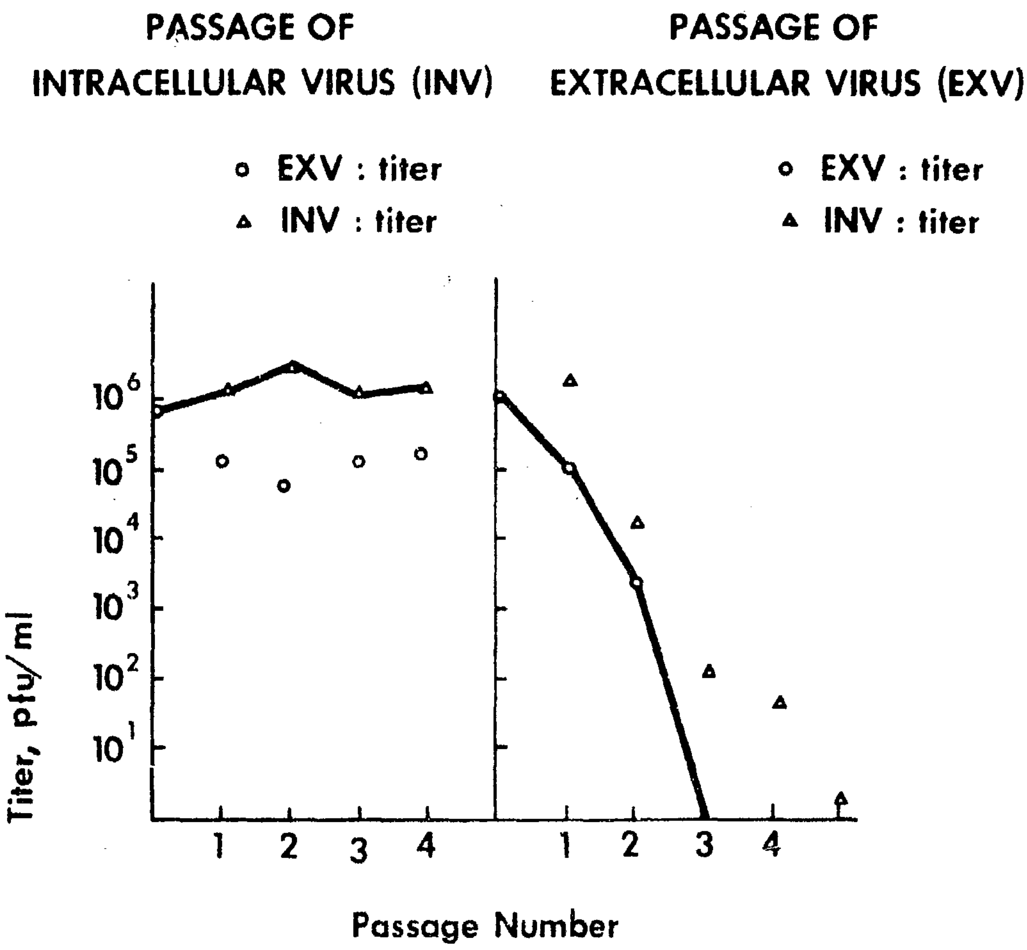

Figure 1. Comparison of Titers of Passed INV and Passed EXV.

TABLE I. TEST FOR INTERFERENCE BY EXTRACELLULAR VIRUS (EXV) PREPARATIONS

	Treatment of GPL Monolayers[a]	Inoculum Titer	First Passage Titer	Second Passage Titer
1.	Not treated	EXV 2.7×10^5	3.2×10^4	4.6×10^3
2.	Not treated	INV 3.3×10^7	3.3×10^7	1.8×10^8
3.	EXV 2.7×10^5	INV 3.3×10^7	1.5×10^7	1.5×10^8
4.	Super. of Centr. EXV 1.5×10^4	INV 3.3×10^7	4.9×10^6	1.4×10^8
5.	Not treated	Super. from Centr. EXV + INV[b] 3.4×10^8	2.4×10^7	5.0×10^8
6.	EXV 2.7×10^5	INV + stale growth medium[b] 2.8×10^7	3.9×10^7	6.2×10^7

a. 24 hours at 35°C.
b. Held 24 hours before placement on cells.

The first line in Table I shows the results with the extracellular virus control; that is, a decline in titer occurs when extracellular virus is inoculated into untreated cultures. The second line shows results with the intracellular virus control; there is an increase in titer when such virus populations are inoculated into untreated cultures.

Lines 3 and 4 show that cultures inoculated with intracellular virus continue to increase in titer even though the cultures were previously treated up to 24 hours with either uncentrifuged extracellular virus or the supernatant from a centrifuged extracellular virus preparation. The use of the latter two preparations was a test for either interfering virus or a "soluble" interferon-like substance.

Lines 5 and 6 show that intracellular virus previously incubated in the presence of the supernate from a centrifuged extracellular virus preparation or in stale growth medium continued to yield increased amounts of virus when infecting untreated cultures previously treated with extracellar virus. In brief, the results indicate that no autointerference by virus or by an interferon-like substance could be demonstrated.

Our next experiments were designed to determine whether the change from passageability to nonpassageability involved a phenotypic or a genotypic change in the virus. From three different passage levels of extracellular virus, intracellular virus was prepared and its passage attempted. Figure 2 shows that even when the extracellular virus was at a very low level, the intracellular virus from this passage level, on continued passage, increased in titer and passed successfully. Notice that before titers started to rise, there was an interval during which no significant increase or decrease in titer occurred, as though some type of "priming" were necessary. This phenomenon will be investigated further.

Our next experiment was the reverse of the one just discussed. We took extracellular virus from different passage levels of intracellular virus to see if the supernatant or extracellular virus preparations could be passed. Figure 3 shows the result of our attempt to pass extracellular virus from the seventh passage of intracellular virus. Passages were made at approximately 24-hour intervals. This extracellular virus preparation, like any other, could not be passed efficiently. Thus, the combined results shown in Figures 3 and 4 indicate that upon release through the cell membrane, a temporary mass phenotypic change occurs in the virus populations that are found in the supernatant fluid. This formally resembles a host-induced modification of virus, except that one cell host instead of two distinguishes between the two virus phenotypes.

The next series of experiments were designed to determine at which stage in the infectious process the passageability differences between intracellular and extracellular virus were reflected. Table II shows results relating to adsorption and penetration of virus in HeLa cells. HeLa cells were chosen for this particular experiment because adsorption and penetration could be measured most precisely and most directly by enumeration of hyperplastic foci. Furthermore, preliminary experiments indicated that the same phenomenon relating to passageability of variola virus occurred in HeLa cells as well as in GPL cells. In this experiment, replicate monolayers of HeLa cells were inoculated with intracellular and extracellular virus preparations and were maintained at 4°C for about one hour. These were then washed and brought to 37°C for the time intervals indicated. At the end of each time interval, antiserum was added to one-half the plates to stop further adsorption and thus allow a measure of the rate and extent of virus penetration. The antiserum was removed by washing after one-half hour and growth medium was added. Essentially, those plates not treated with antiserum measured adsorption plus penetration.

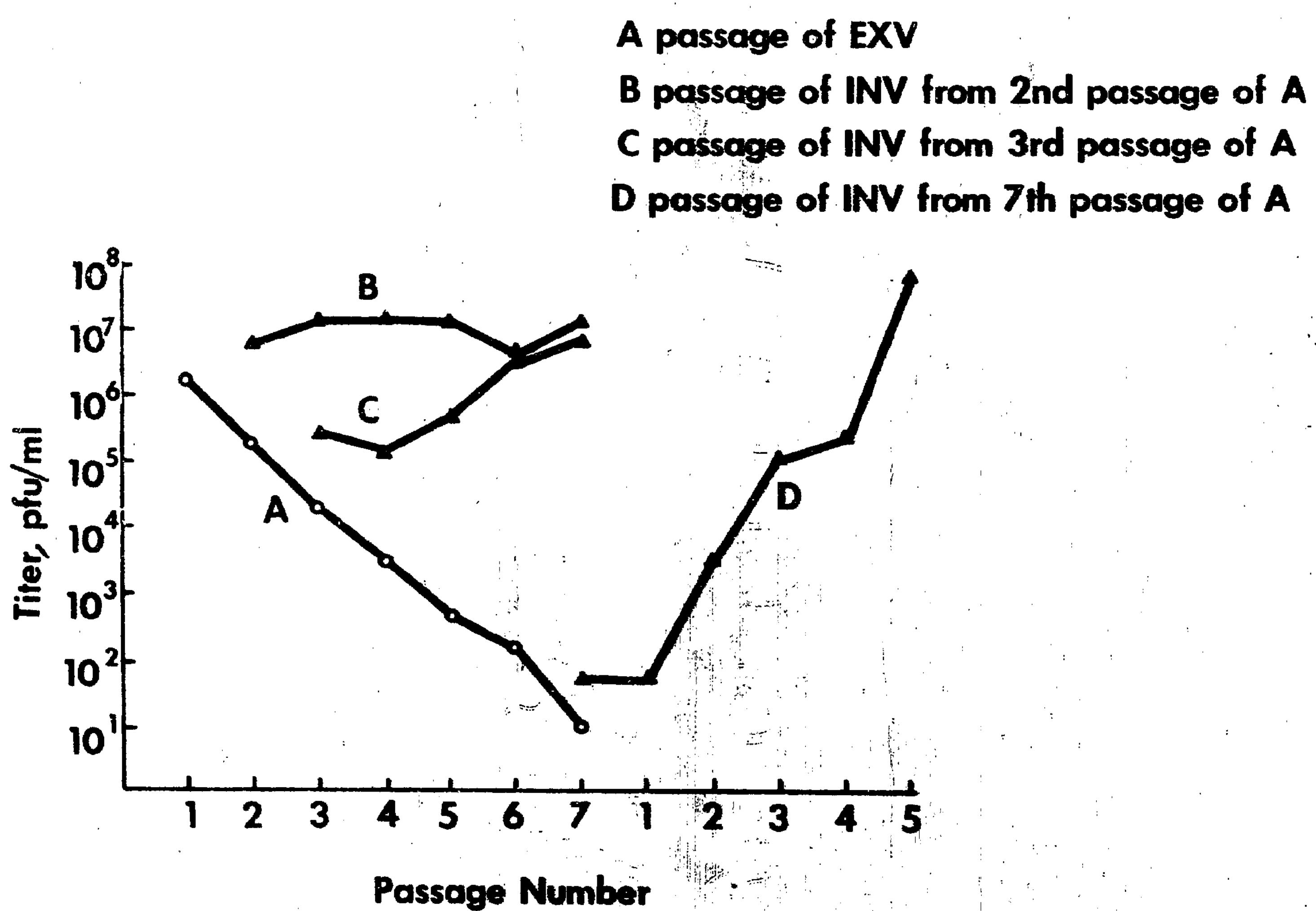

Figure 2. Passage of Intracellular Virus (INV) Taken at Different Passage Levels of Extracellular Virus (EXV).

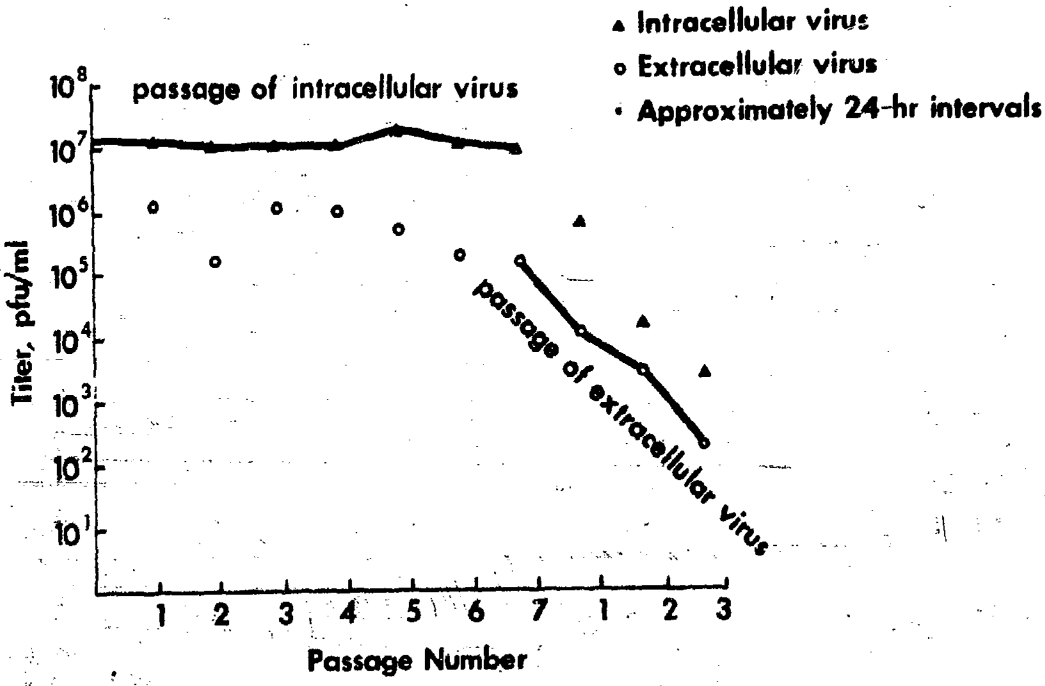

Figure 3. Rapid Passage* of Intracellular Variola Virus Followed by Passage of Extracellular Virus.

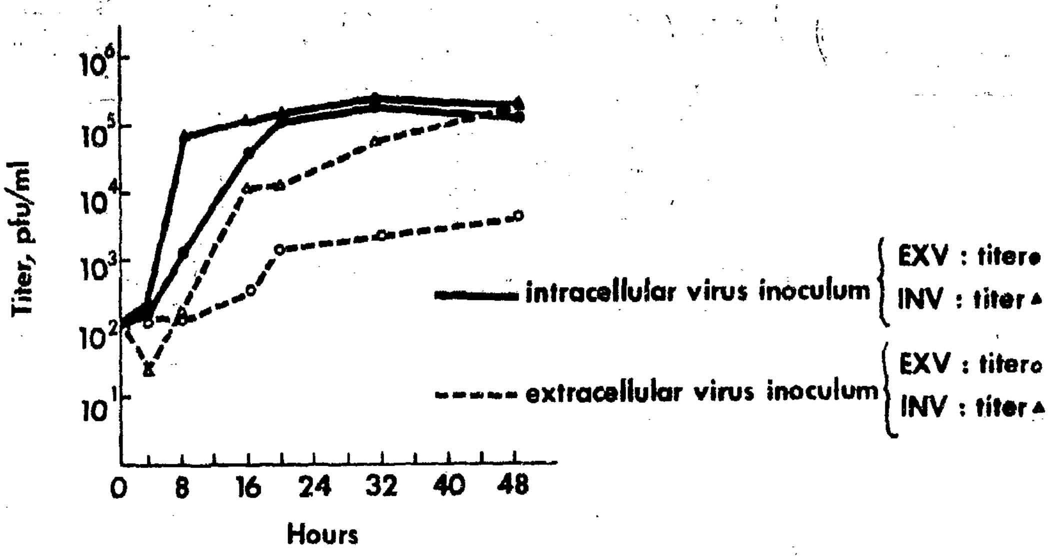

Figure 4. Growth and Release of Extracellular (EXV) and Intracellular (INV) Variola Virus from GPL Cells.

TABLE II. DIFFERENCES IN ADSORPTION AND PENETRATION BETWEEN INTRACELLULAR (INV) AND EXTRACELLULAR (EXV) VARIOLA VIRUS[a]

Exp. No.		Hour Antiserum Added 0	0.5	1	1.5	Final Fold Increase Over 0 Hour
1	EXV	120	237	196	231	1.9
	EXV plus antiserum[b]	36	117	105	141	4.0
	INV	169	312	328	388	2.3
	INV plus antiserum	19	132	209	270	13.0
2	EXV	23	26	30	61	2.4
	EXV plus antiserum	8	25	22	29	3.6
	INV	92	132	166	152	1.6
	INV plus antiserum	4	46	81	140	25.0

a. As measured by hyperplastic foci on monolayers of HeLa cells.
b. Convalescent monkey antiserum to egg seed virus.

The results in Table II indicate that there is very little difference between adsorption of intracellular and extracellular virus, but that the rate and extent of penetration by intracellular virus significantly exceeds that of extracellular virus.

Figure 4 shows results indicating that in GPL cells, extracellular virus has a longer latent phase, multiplies more slowly, and is released much less efficiently than intracellular virus.

We are continuing our work to define more precisely the qualitative differences between intracellular and extracellular virus in immunological, biochemical, and biophysical terms. The results of our first four experiments using immunological techniques are shown in Table III. The last column shows that convalescent monkey antiserum taken from a monkey infected with an egg seed preparation of the virus neutralizes intracellular virus more extensively than extracellular virus. These differences are significant in view of the low coefficient of variation (ca. 10) that can be obtained with single virus preparations by this method of assay. We hope to extend these results by comparing intracellular and extracellular virus in their densities, net charge, and morphological particle types as observed in the electron microscope.

TABLE III. DIFFERENCES IN NEUTRALIZATION BETWEEN EXTRACELLULAR (EXV) AND INTRACELLULAR (INV) VARIOLA VIRUS BY ANTISERUM

Exp. No.		Number[a] of Foci	Reduction, per cent
1	EXV	120	70
	EXV plus antiserum[b]	36	
	INV	169	91
	INV plus antiserum	19	
2	EXV	23	65.2
	EXV plus antiserum	8	
	INV	92	95.6
	INV plus antiserum	4	
3	EXV	101	34.6
	EXV plus antiserum	66	
	INV	166	88.5
	INV plus antiserum	19	
4	EXV	31	35.5
	EXV plus antiserum	20	
	INV	116	91.4
	INV plus antiserum	10	

a. Average of replicate plates.
b. Convalescent monkey antiserum to egg seed virus.

IV. SUMMARY

In summary, our results show the following:

(a) The change from efficient passageability to poor passageability of variola virus that occurs when it is released from its intracellular environment to the supernatant medium involves a reversible phenotypic change.

(b) The qualitative difference between intracellular and extracellular virus preparations is reflected in differences between them in most of the phases of the growth cycle in guinea pig lung cells. Essentially, the end result is that, in contrast to intracellular virus, when extracellular virus is used to infect GPL cells, a very small percentage of it is released for the infection of new cells.

(c) The intracellular virus was also found to be neutralized more extensively by convalescent monkey antiserum than was extracellular virus.

LITERATURE CITED

1. Pirsch, J.B.; Mika, L.A.; Purlson, E.H.; and Brown, A. "Factors influencing the passage of variola virus in tissue cultures," Bacteriol. Proc. V133, 1962.

2. Pirsch, J.B., and Purlson, E.H. "A tissue culture assay for variola virus based upon the enumeration of hyperplastic foci," J. Immunol. 89:632-637, 1962.